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PB-284 764

# The Effect of Ozone on Human Immunity: In vitro Responsiveness of Lymphocytes to Phytohemmagglutinin

U.S. Health Effects Research Lab, Research Triangle Park, N C

15 Mar 76

**TECHNICAL REPORT DATA**
*(Please read Instructions on the reverse before completing)*

| 1. REPORT NO. | 2. | 3. PB 284 764 |
|---|---|---|
| EPA-600/J-78-025 | JOURNAL ARTICLE | |

**4. TITLE AND SUBTITLE**
The Effect of Ozone on Human Immunity: In Vitro Responsiveness of Lymphocytes to Phytohemmagglutinin

**5. REPORT DATE**

**6. PERFORMING ORGANIZATION CODE**

**7. AUTHOR(S)**
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**8. PERFORMING ORGANIZATION REPORT NO.**

**9. PERFORMING ORGANIZATION NAME AND ADDRESS**
Clinical Studies Division
Health Effects Research Laboratory
Office of Research and Development
Research Triangle Park, N.C. 27711

**10. PROGRAM ELEMENT NO.**
1AA601

**11. CONTRACT/GRANT NO.**

**12. SPONSORING AGENCY NAME AND ADDRESS**
Health Effects Research Laboratory RTP, NC
Office of Research and Development
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

**13. TYPE OF REPORT AND PERIOD COVERED**

**14. SPONSORING AGENCY CODE**
EPA 600/11

**15. SUPPLEMENTARY NOTES**
Published in: Arch. Environ. Health 33(2):59-63, 1978

**16. ABSTRACT**

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The significance of the suppression of T-cell response noted in this study is that (1) if continuous exposures to ozone are shown to induce an immunosuppressed state for a significant time period, an important factor in carcinogenesis might be elucidated; (2) immunosuppression may cause a progression of an already present tumor; (3) immunosuppression may enable endogenous latent infection such as tuberculosis to reactivate; and (4) immunosuppression may explain in part the relationship between chronic oxidant air pollution and influenza-like illnesses in population.

**17. KEY WORDS AND DOCUMENT ANALYSIS**

| a. DESCRIPTORS | b. IDENTIFIERS/OPEN ENDED TERMS | c. COSATI Field/Group |
|---|---|---|
| ozone<br>immunity<br>lymphocytes<br>environmental tests | | 06 F, T |

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NATIONAL TECHNICAL INFORMATION SERVICE
U. S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

| 18. DISTRIBUTION STATEMENT | 19. SECURITY CLASS *(This Report)* | 21. NO. OF PAGES |
|---|---|---|
| RELEASE TO PUBLIC | UNCLASSIFIED | 7 |
| | 20. SECURITY CLASS *(This page)* | 22. PRICE |
| | UNCLASSIFIED | |

EPA Form 2220-1 (9-73)

EPA-600/J-78-025
JOURNAL ARTICLE

# The Effect of Ozone on Human Immunity: In Vitro Responsiveness of Lymphocytes to Phytohemmagglutinin

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**ABSTRACT**

Peripheral blood lymphocytes from 20 human subjects exposed to 784 $\mu g/m^3$ ozone for 4 hours, and from 11 subjects exposed to clean air for the same length of time were studied for in vitro responsiveness to phytohemagglutinin (PHA). Thymus-derived (T) lymphocyte response to PHA (normal response is proliferation of lymphocytes) was significantly suppressed ($P < .01$) in samples obtained immediately after subjects' exposure to ozone. Recovery of response occurred 2 weeks postexposure. Responses were unchanged in subjects exposed to clean air.

Existing studies suggest that ozone exposure may generate free radicals or other reactive molecules or both, that could be responsible for immediate changes in metabolic events leading to blockage or inhibition of deoxyribonucleic acid (DNA) synthesis in T lymphocytes as shown in this study. It is possible that some prerequisite to active cell metabolism such as ribonucleic acid (RNA) may be impaired by ozone exposure.

The significance of the suppression of T-cell response noted in this study is that: (1) if continuous exposures to ozone are shown to induce an immunosuppressed state for a significant time period, an important factor in carcinogenesis might be elucidated; (2) immunosuppression may cause a progression of an already present tumor; (3) immunosuppression may enable endogenous latent infection such as tuberculosis to reactivate; and (4) immunosuppression may explain in part the relationship between chronic oxidant air pollution and influenza-like illnesses in population.

OZONE, a highly reactive gas, is a natural constituent of the upper atmosphere and is a major component of environmental photochemical smog derived from primary air pollutants and solar irradiation.[1] Its possible effect on cells of the human immune systems is poorly understood. Evidence from animal studies has demonstrated that ozone reacts in a random fashion with proteins to produce a heterogenous antigen which will stimulate an antibody response,[2] depress various intracellular hydrolytic enzymes and reduce cellular viability,[3,4] and increase susceptibility to bacterial infection.[5-7]

Ozone-induced alterations in normal lymphocyte function may underlie some of these reported alterations in host response to infectious agents. With this possibility in mind, a study was designed to examine the in vitro response to phytohemagglutinin (PHA) of peripheral blood lymphocytes from healthy humans exposed to low levels of ozone under controlled environmental conditions. Lymphocyte response to PHA is presently the most satisfactory in vitro index of thymus-derived lymphocyte (T cell) function, and is thus an important tool in the clinical assessment of cell-mediated immunity.[8] This report discusses the responsiveness of lymphocytes before and at several time periods after a single 4-hour exposure to 784 $\mu g/m^3$ (0.4 ppm) ozone. In addition, PHA dose-response measurements derived from lymphocyte responses of normal individuals and of subjects exposed to clean air are included.

## Materials and Methods

### *Subject Selection*

Male volunteers between the ages of 19 and 27 were studied after informed consent was obtained. All subjects completed a comprehensive medical questionnaire and were examined by a physician. None of the subjects smoked or had a history of cardiac disease, allergy, or infections. No subjects had used any medications for at least 1 week prior to testing.

### *Baseline Study*

A baseline study was conducted to establish a normal lymphocyte response curve to various concentrations of PHA. Eighteen individuals participated in this study. Blood was drawn at 8:00 A.M. Fourteen of these subjects participated later in ozone and 4 in clean air exposure studies.

### *Exposure Studies*

Exposures to ozone or clean air took place in an airtight, 2.4 m × 2.4 m × 2.4 m chamber constructed from 1.3-cm-thick acrylic plastic.[2,5] Subjects were seated for 4 hours, except during two 15-minute exercise periods on a bicycle ergometer at a workload of 700 kg m/min—an exercise level that produced an approximate doubling of heart rate. An electrocardiogram (ECG) was monitored continuously. The chamber was operated at a slightly negative pressure. Ozone was generated from oxygen



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(OREC ozone generator) and injected into the ambient air supply duct at a low pressure point downstream from an orifice plate used to measure the flow of the ambient laboratory air into the chamber. The air-ozone mixture was dispersed through a perforated grill after mixing. Ozone levels in the chamber were sampled continuously with two Bendix chemiluminescence analyzers. The concentration of ozone was maintained between 686 and 823 $\mu g/m^3$ (0.35 and 0.42 ppm) at all times and was uniform in all parts of the chamber. During all exposures ambient air was continuously monitored for ozone, sulfur dioxide, nitrogen oxides, carbon monoxide, and total suspended particulates. Concentrations of these pollutants were extremely low at all times. The chamber temperature was maintained at 21 to 24°C and relative humidity at 40 to 60%.

Blood was drawn before each exposure at 8:00 A.M., 4 hours after each exposure, between 10:00 A.M. and 12:00 A.M. on day three, and 2 weeks after exposure to ozone.

*Lymphocyte Preparation*

Venous blood from each volunteer was collected into sterile, siliconized tubes prepared with heparin, 20 U/ml (Lipo-Hepin, Riker Labs., Inc.). Lymphocytes were separated by the Methocell/Hypaque (M-H) method described previously by Main and Jones[9] and modified by Silverman (personal communication). Briefly, a 1.5% solution of methylcellulose (Fisher Scientific Co., grade U.S.P., 100 centipoise, Lot No. 714360) was dissolved in boiling distilled water and sterilized by autoclaving. This solution and 50% Hypaque (sodium diatrizoate, 50% sterile solution; Winthrop Labs) were mixed in a ratio of 16:10, with a specific density of 1.080. A 5.0-ml portion of M-H mixture was dispersed into each of six siliconized, sterile, 16- × 150-mm glass tubes; each portion was carefully overlayered with 2.5 ml of blood and incubated at 37°C for about 60 minutes. The overlay of leukocyte-rich plasma was removed and centrifuged at 800 rpm for 20 minutes at 4°C. The cells were then washed twice with Hanks' balanced salt solution and suspended to a concentration of $10^6$ cells/ml in an RPMI-1640 medium containing L-glutamine (Grand Island Biological Co.), 100 U of penicillin per ml, 100 $\mu g$ of streptomycin per ml, and heat-inactivated fetal calf serum (20% of the final volume). The cell suspensions of subjects tested contained from 56% to 83% (mean of 74%) lymphocytes, with a viability of greater than 98% on trypan blue exclusion. The monocyte concentration was 2 to 4%.

*Lymphocyte Transformation*

Twelve culture tubes (Falcon No. 2003) were prepared for each subject. All tubes contained 1.0 ml of lymphocyte suspension ($10^6$ cells). Three tubes contained a 1.0 ml suspension medium without PHA and three sets of triplicate tubes contained 1.0 ml portions of purified PHA (Burroughs Wellcome, Co.; PHA Lot 8869) at concentrations of 2, 20, and 200 $\mu g$/ml per $5 \times 10^5$ lymphocytes, respectively.[10] The tubes were loosely capped and incubated at 37°C in 5% carbon dioxide in a humidified atmosphere for 3 days. The cells in each tube were then pulsed for 6 hours with [2-$^{14}$C] thymidine (0.1 $\mu$Ci in 0.5 ml, specific activity 54.6 mCi/nmole; New England Nuclear).[11] The reaction was stopped by placing tubes in an ice bath. Lymphocytes were recovered by cold centrifugation and washed in saline at 4°C. DNA was precipitated by the addition of trichloracetic acid dissolved in Soluene-100 (Packard), and washed into glass scintillation vials with scintillation fluid (Liquifluor, New England Nuclear). The amount of [2-$^{14}$C] thymidine that had been incorporated into DNA in each culture was counted in a Packard liquid scintillation spectrometer for 10 minutes. The mean of each set of three tubes was determined and expressed as counts per minute (cpm) per $5 \times 10^5$ lymphocytes for statistical analysis.

*Statistical Analyses*

Computations were made by analyses of variance with repeated measurements.[12] In order to satisfy the assumptions of this method, the DNA counts were transformed by computing $\log_{10}$ of the counts. Three hypotheses were tested: the first hypothesis was that there was no difference in the mean of the transformed data before exposure and 4 hours after exposure; the second and third hypotheses incorporated in the author's 72-hour and 2-week results instead of 4-hour results.

**Results**

*Baseline Study*

PHA-induced lymphocyte transformation responses were assessed in 18 normal subjects (Fig. 1). Increasing concentrations of PHA gave a dose-response curve with peak responses at 2 $\mu g$/ml (26,100 ± 5,700) and 20 $\mu g$/ml (26,400 ± 3,900). A considerably lower response was seen in cultures stimulated with 200 $\mu g$/ml of PHA. Unstimulated lymphocytes incorporated [2-$^{14}$C] thymidine to give a mean of 280 ± 80. Since the peak stimulation occurred at 2 $\mu g$/ml and 20 $\mu g$/ml, these PHA concentrations were chosen for the remaining studies.

*Air Exposure Study*

Lymphocyte response to PHA was studied in eleven subjects before and after a 4-hour control exposure to air in the chamber. The subjects followed the same exercise protocol as did ozone subjects. No statistically detectable differences were observed in mean responses to 2 $\mu g$/ml and 20 $\mu g$/ml of PHA in cultures taken before and immediately after the exposure. Unstimulated cultures had no detectable differences in DNA uptake of [2-$^{14}$C] thymidine over the 4-hour exposure period.

*Ozone Exposure Study*

Figure 2 and Figure 3 illustrate the means ± statistical error of lymphocyte responses to 2 $\mu g$/ml and 20 $\mu g$/ml, respectively, of PHA in cultures from 20 subjects before and 4 hours, 72 hours, and 2 weeks after exposures to ozone. The mean lymphocyte response to 2 $\mu g$/ml PHA was significantly suppressed ($P < .01$) in samples obtained

immediately postexposure, when compared with those obtained preexposure (difference between 29,700 ± 5,300 and 20,900 ± 5,700, respectively; Fig. 2). Cultures stimulated with 20 μg/ml PHA also displayed a decreased responsiveness (decrease from 23,900 ± 3,400 to 20,900 ± 5,700; Fig. 3), but the difference was not statistically significant. A slight recovery of lymphocyte response with PHA dosage of 2 μg/ml was observed in cultures obtained 72 hours after exposure. Cultures stimulated with 20 μg/ml PHA showed no recovery at 72 hours. The DNA counts with both PHA values at 2 weeks were not significantly different from preexposure levels. No significant differences were seen in DNA counts of unstimulated cultures at 0 hours, 4 hours, 72 hours, and 2 weeks; the corresponding figures were 240 ± 50, 300 ± 270 ± 50, and 290 ± 70, respectively.

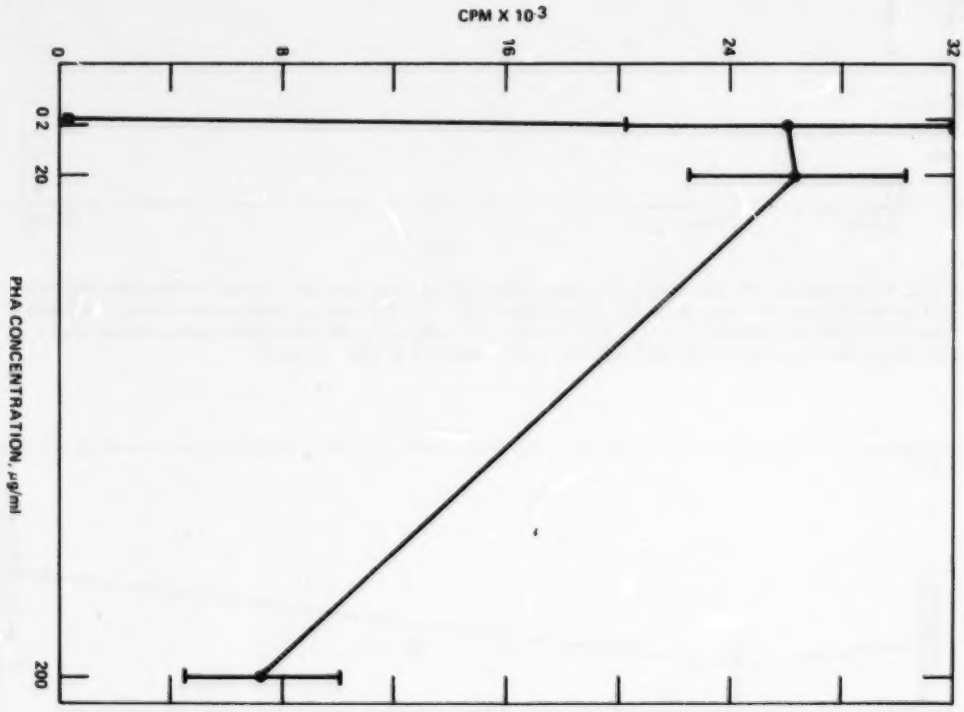


**Fig. 1. Dose-response curve for PHA-treated lymphocytes from normal human subjects. Proliferative response was measured as in vitro DNA uptake of [2-$^{14}$C] thymidine in lymphocyte cultures. Each data point ( ● ) is the mean uptake in cpm ($\times 10^{-3}$) per $5 \times 10^5$ cells, for lymphocytes from eighteen subjects; ( I ) is the SE of the mean.**

## Comment

Our studies show that one 4-hour exposure to 748 μg/m$^3$ ozone suppresses the PHA responsiveness of T cells in peripheral blood from normal human subjects. The maximal suppression of response was observed immediately after the exposure. The responses returned to normal in 2 weeks.

The mechanism of ozone action on lymphocytes is at present open to speculation. The existing studies suggest that ozone is radiomimetic and that the mechanism of its action may be, in part at least, through free radicals, a concept hypothesized for ionizing radiation.[13,14] As do X-rays, ozone produces chromosomal aberrations[15] and, like ultraviolet (UV) irradiation, retardation in deoxygenation of hemoglobin in capillaries.[16] It would appear that generation and propagation of free radicals or other reactive molecules, or both, by ozone could result in changes in nucleic acid synthesis, alteration in protein structure, and interference in the function of cell and organelle membranes. The suppression of PHA-responsiveness of lymphocytes following exposure would

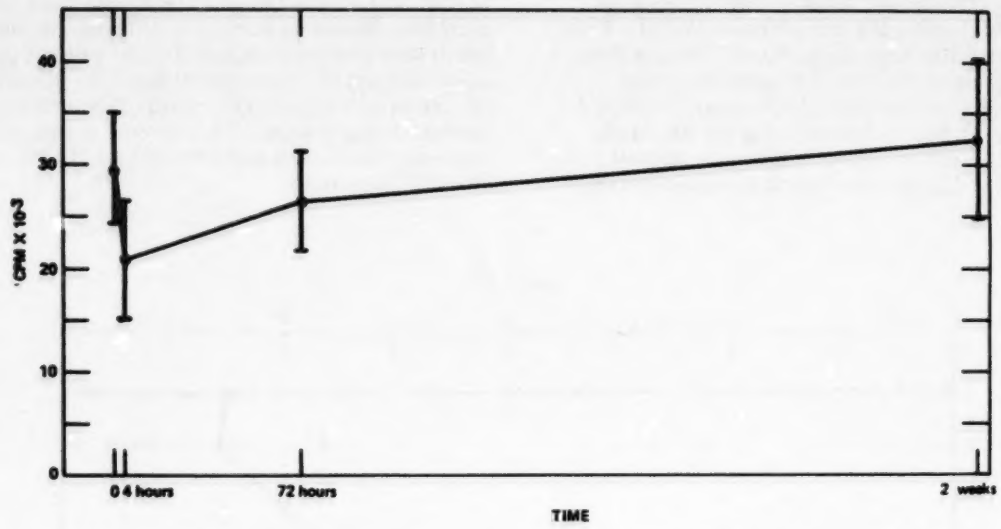


Fig. 2. Suppressed lymphocyte responses at several time intervals after exposure to ozone. Proliferative response to 2 μg/ml PHA was measured as in vitro DNA uptake of [2-$^{14}$C] thymidine in lymphocyte cultures. Each data point ( ● ) is the mean uptake in cpm ($\times 10^{-3}$) per $5 \times 10^5$ cells, for lymphocytes from twenty individuals; ( I ) is the SE of the mean. Significant suppression ($P < .01$) was seen 4 hr after exposure.

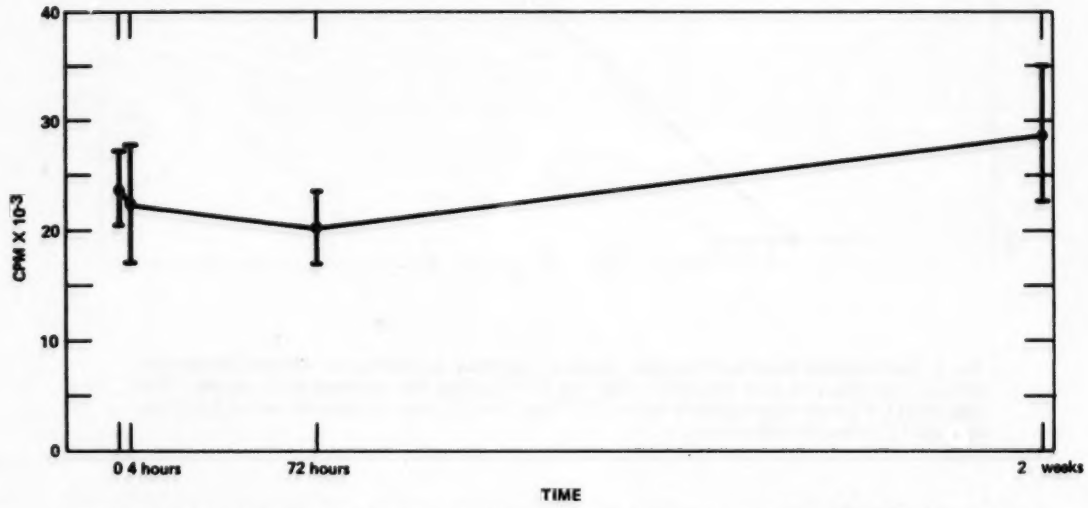


Fig. 3. Suppressed lymphocyte responses at various time intervals after exposure to ozone. Proliferative response to 20 μg/ml PHA was measured as in vitro DNA uptake of [ 2-$^{14}$C] thymidine in lymphocyte cultures. Each data point ( ● ) is the mean uptake in cpm ($\times 10^{-3}$) per $5 \times 10^5$ cells, for lymphocytes from twenty individuals; ( I ) is the SE of the mean.

then indicate that ozone had a pronounced immediate effect on the normal proliferation response of the cell, leading to blockage of DNA synthesis, similar to the effect of UV irradiation.[17] Since the transforming lymphocytes pass through various stages of interface activity prior to reaching DNA synthesis,[18] it is possible that some factor prerequisite to active cell metabolism was impaired by ozone. The obvious candidate in this context is believed to be RNA, as the nucleic acids are considered to be the most UV-sensitive cell component.[19] Studies in mice show that PHA stimulates primarily the more mature T lymphocytes.[20] Thus, the suppressed response to PHA after the exposure may indicate that ozone impairs a more mature lymphocyte subpopulation.

The 784 $\mu g/m^3$ concentration of ozone (0.4 ppm) utilized in these studies closely approximates ozone levels found on high smog days in certain areas of Southern California, where ozone levels approaching 1176 $\mu g/m^3$ (0.6 ppm) for 1 to 2 hours have been measured.[21] Since the lymphocyte response in vitro closely approximates the normal function of cellular immunity in vivo[22,23] the continuous daily exposures to moderate concentrations of ozone may impair normal immunological functions of B lymphocytes as a result of impairment to T cells.

The present study is the first to evaluate T-lymphocyte response to PHA in humans exposed to low levels of ozone. The significance of the suppression of T-cell response noted in this study is that: (1) if continuous exposures to ozone are shown to induce an immunosuppressed state for a significant period of time, an important factor in carcinogenesis might be elucidated, as there is ample evidence that the immunosuppressed state is now associated with higher incidence of malignancy;[24] (2) immunosuppression may cause a progression of an already present tumor, and enable an endogenous latent infection such as tuberculosis to reactivate; and (3) immunosuppression may explain, in part, the relationship between air pollutants and respiratory infection in man.

To approximate the actual exposure experience and its relationship to the immune mechanisms in humans, additional investigations are in progress. These include one 2-hour exposure to 1176 $\mu g/m^3$ (0.6 ppm) ozone and characterization of T- and B-cell populations based on their stimulability by mitogens, surface membrane receptors and immunoglobulin synthesis. It is apparent that information gained from studies such as these will provide a more precise understanding of immunosuppressive effects of oxidant air pollution at the cellular level.

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The authors are grateful to the Audio Visual Media for preparing the graphs; Beverly Tilton for editing; and Kathy Mayfield for typing the manuscript.

Mention of a commercial product does not imply endorsement by the United States Government.

These data have been presented in part at the 76th Annual Meeting of the American Society for Microbiology in Atlantic City, N.J., May 1976.

The protocol was approved by the Committee for Protection of Human Subjects of the University of North Carolina, School of Medicine, Chapel Hill, N.C.

Submitted for publication March 15, 1976; revised; accepted November 3, 1976.

Article copies are available from: Mirdza L. Peterson, Ph.D., Clinical Microbiology/Immunology Laboratory, Clinical Studies Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Chapel Hill, North Carolina 27514.

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## REFERENCES

1. U.S. Department of Health, Education, and Welfare, National Air Pollution Control Administration. 1970. *Air quality criteria for photochemical oxidants.* Publication No. AP-63. Washington, D.C.
2. Scheel, L. D.; Dobrogorski, O. J.; Mountain, J. T.; Svirbely, J. L.; Stokinger, H. E. 1959. Physiologic, biochemical, immunologic and pathologic changes following ozone exposure. *J Appl Physiol* 14:67-80.
3. Alpert, S. M.; Gardner, D. E.; Hurst, D. J.; Lewis, T. R.; Coffin, D. L. 1971. Effects of exposure to ozone in defensive mechanisms of the lung. *J Physiol* 31:247-52.
4. Hurst, D. J.; Gardner, D. E.; and Coffin, D. L. 1970. Effect of ozone on acid hydrolases of pulmonary alveolar macrophages. *J Reticuloendothel Soc* 8:288-300.
5. Coffin, D. L., and Gardner, D. E. 1972. Interaction of biolgical agents and chemical air pollutants. *Ann Occup Hyg* 15: 219-35.
6. Stokinger, H. E., and Coffin, D. L. 1968. Biologic effects of air pollutants. In *Air pollution, vol. 1.* 2nd ed. A. C. Stern (ed.), New York: Academic Press. pp. 445-68.
7. Miller, S., and Ehrlich, R. 1958. Susceptibility to respiratory infections of animals exposed to ozone: Susceptibility to *Klebsiella pneumoniae. J Infect Dis* 103:145-49.
8. Fitzgerald, M. G. 1972. A satisfactory quantitative test of lymphocyte response to phytohemagglutinin for the definition of normal control values and recognition of immunological defects. *J Clin Path* 25:163-68.
9. Main, R. K., and Jones, M. J. 1968. Mixed leucocyte interaction involving mouse strains of strong and weak allogeneic specificities. *Nature* 218:1251-52.
10. Fitzgerald, M. G. 1971. The establishment of a normal human population dose-response curve for lymphocytes cultured with PHA (phytohemagglutinin). *Clin Exp Immunol* 8:421-25.
11. Valentine, F. 1971. Lymphocyte transformation: The proliferation of human blood lymphocytes stimulated by antigen in vitro. B. R. Bloom and P. R. Glade (ed.). In *vitro methods in cell-mediated immunity,* pp. 443-54. New York: Academic Press.
12. Winer, B. J. 1963. *Statistical principles in experimental design.* New York: McGraw Hill.
13. Brinkman, R.; Lamberts, H. B.; and Veninga, T. S. 1964. Radiomimetic toxicity of ozonised air. *Lancet* 1:133-36.
14. Stokinger, H. E. 1965. Ozone toxicology: A review of research and industrial experience. *Arch Environ Health* 10:719-31.
15. Zelac, R. E.; Cromroy, H. L.; Bolch, W. E., Jr.; Dunavant, B. G.; Bevis, H. A. 1971. Inhaled ozone as a mutagen. I. Chromosone aberrations induced in Chinese hamster lymphocytes. *Environ Res* 4:262-82.
16. Brinkman, R., and Lamberts, H. B. 1958. Ozone as a possible radiomimetic gas. *Nature* 181:504-5.
17. Lindahl-Kiessling, K., and Safwenberg, J. 1971. Inability of U.V.-irradiated lymphocytes to stimulate allogeneic cells in mixed lymphocyte cultures. *Int Arch Allergy* 41:670-78.
18. Chalmers, D. G.; Cooper, E. H.; Coulson, A. S.; Inman, D. R.; Topping, N. E. 1966. Immunologically stimulated human perperipheral blood lymphocytes in vitro . I. Morphological changes. *Int Arch Allergy* 30:83-96.
19. Smith, K. C., and Hanawalt, T. C. 1969. *Molecular photobiology.* New York: Academic Press.
20. Stobo, J. D., and Paul W. E. 1972. Functional heterogeneity of murine lymphoid cells. II. Acquisition of mitogen responsiveness and of Oantigen during the ontogeny of thymocytes and "T" lymphocytes. *Cell Immunol* 4:367-80.
21. Environmental Protection Agency. 1973. *Monitoring and air quality trends report.* Publication No. EPA-450/1-74-007. Research Triangle Park, North Carolina.
22. Bloom, B. R. 1971. In vitro approaches to the mechanism of cell mediated immune reactions. *Adv Immunol* 13:101-208. p. 111.
23. Nakamura, R. M.; Bell, C. A.; Beathard, G. A.; Daniels, J. C. Egan, M. L.; Ritzman, S. E.; Sakai, H.; Townsend, C. M.; Tucker, E. S.; Vyvial, T. M. 1974. *Immunopathology. Clinical laboratory concepts and methods.* Boston: Little, Brown and Company. pp. 13-17.
24. Good, R. A. 1972. Relations between immunity and malignancy. *Proc Nat Acad Sci* 69:1026-32.
25. Strong, A. A.; Penley, R.; Knelson, J. H. 1977. *Human exposure system for controlled ozone atmospheres.* U.S. Environmental Protection Agency. EPA-600/1-77-048. North Carolina: Research Triangle Park.